

CHAPTER IV RESEARCH METHOD

4.1 Research Field

This research was in the field of genetics and ophthalmology.

4.2 Research Period and Location

This research had been done from July to December 2014 in Palembang City, South Sumatera and Centre of Biomedical Research (CEBIOR) Faculty of Medicine Diponegoro University, Semarang, Central Java, Indonesia.

4.3 Research Design

The design of this research was cross sectional study.

4.4 Material

4.4.1 Population

The population of this study was Indonesian people from South Sumatera ethnic group.

4.4.2 Sampling

Samples were selected with random sampling method from Muhammadiyah Palembang University students and staffs.

4.4.2.1 Subject Selection

Subject for this study had been going through history taking and limited physical examination i.e ocular region, visual acuity measurement with Snellen

chart, subjective test with trial lens, and corneal curvature measurement with manual keratometer (Takagi, Japan).



Figure 6. Manual keratometer Takagi, Japan
Source: private documentation.

Inclusion criteria for case subject was:

1. Male and female from South Sumatera tribes with visual acuity $<6/6$ and gave positive response with spherical concave lens on both eyes.
2. Age on examination was 18-40 years old.
3. Subject was literate person.

Inclusion criteria for control subject was:

1. Male and female from South Sumatera tribes with visual acuity 6/6 and gave negative response with spherical concave and convex lens on both eyes.
2. Age on examination was 18-40 years old.
3. Subject was literate person.

Exclusion criteria for this study are:

1. Subject with early-adult and late adult onset of myopia.

2. Subject with myopia only on one eye.
3. Subject who had history of ophthalmic surgery.
4. Subject who had ophthalmic disease during subject recruitment such as cataract, keratitis, conjunctivitis.

4.4.2.2 Sample Estimation

For cross sectional study, the formula to estimate the minimum sample requirement was⁵⁸:

$$n = \frac{(Z_{\alpha})^2 pq}{d^2}$$

With:

n = minimum sample requirement

p = early-onset myopia prevalence in Palembang city, Indonesia (46.7%)⁹

q = 1-p

d = error limit or absolute precision (10%)

Z_{α} over 2 for α 5% = 1.96

From the formula above, the minimum sample requirement for this study was 96 subjects. Total 100 subjects were taken as sample for this study.

4.5 Research Method

4.5.1 Questionnaire Administration

All subjects in this study had answered the questionnaire which contained 23 questions about five main risk factors in this study, categorized as

sex, family history of myopia, near work activities, outdoor activity and lighting.

4.5.2 SNP Location

Gene sequence reference was taken from the www.ncbi.nlm.nih.gov with accession number NM_006206. This study was designed to investigate 7 SNPs near *PDGFRA* gene as the result of previous Genome Wide Association Study in corneal curvature of Singaporean citizens.¹¹

Table 3. Location of SNPs in *PDGFRA* gene¹¹

SNP	Location
rs7676985	[NC_000004.12:g.54198406G>A]
rs7682912	[NC_000004.12:g.54219453T>G]
rs17084051	[NC_000004.12:g.54221414C>A]
rs2114039	[NC_000004.12:g.54226459T>C]
rs7677751	[NC_000004.12:g.54258293C>T]
rs2307049	[NC_000004.12:g.54263987G>A]
rs7660560	[NC_000004.12:g.54268227G>A]

All of SNPs above were located in introns of *PDGFRA* gene, so this variations could induced alternative splicing i.e exon skipping, alternative 3' splice site activation, alternative 5' splice site activation, intron retention, mutually exclusive splicing.⁵⁹

4.5.3 Primer design

Primers were designed using Primer3Plus software available online at <http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>.

4.5.4 DNA Extraction

The subjects were asked to participate on this research by signing the consent form and giving their DNA sample from buccal swab. DNA samples were extracted using DNA extraction kit. The extraction process followed the factory manual guide.

4.5.5 DNA Amplification

PDGFRA gene was amplified and analyzed using High Resolution Melting Analysis (HRMA) technique using qPCR RotorGeneQ 5Plex HRM (Qiagen, California, USA). Each well consisted of 10 µL of Type-It HRM Master Mix (Qiagen, California, USA, Cat. No. 206542), 10 pmol/ µL forward primer, 10 pmol/ µL reverse primer, 10 ng DNA, and PCR-grade water adjusted until 20 µL total volume in 0.2 µL PCR tube.

The curve analysis had been done using Rotor-Gene ScreenClust HRM Software (Qiagen, California, USA). PCR product were confirmed by looking at the data of fluorescent level against cycle. One sampel had been chosen randomly from each difference graph and proceed to the sequencing process.

4.6 Operational Definition

1. *PDGFRA* gene polymorphism

Definition: Genetic variation which is caused by a change in one

basepair of SNP sequence in 5506457G>A, 55085620T>G, 55087581C>A, 55092626T>C, 55124460C>T, 55130154G>A, and 55134394G>A. The variation will be analyzed by melting curve difference

with qPCR-HRMA and the aberrant graphs will be proceed to sequencing process.

Scale: Nominal

Result: 1. Yes

2. No

2. Early-onset myopia

Definition: Nearsightedness occurs before 20 years old of age, on both eyes.

Scale: Nominal

Result: 1. Yes

2. No

3. Sex

Definition: Physical appearance of female or male.

Scale: Nominal

Result: 1. Male

2. Female

4. Reading in dim light

Definition: Subjective estimation about quality of their room lighting while reading. The estimation will be divided into dim light and bright light.

Scale: Ordinal

Result: 1. Dim light

2. Bright light

5. Near work duration and frequency

Definition: Subjective estimation about duration and frequency of doing near work. Near work in this study means reading, watching television, using computer/tablet/smart phone.

Scale: Ordinal

Result: a. Duration of near work for pleasure

1. < 30 minutes per day
2. 30 minutes - 2 hours per day
3. >2 hours per day

b. Duration of near work for school

1. <8 hours per day
2. 8-13 hours per day
3. >13 hours per day

c. Frequency of near work for pleasure

1. 1-2 times per week
2. 3-5 times per week
3. ≥ 6 times per week

d. Frequency of near work for school

1. 1-2 times per week
2. 3-5 times per week
3. ≥ 6 times per week

6. Outdoor activity duration and frequency

Definition: Subjective estimation about duration and frequency of doing outdoor activity. Outdoor activity in this study means sport, physical exercise, play in the park.

Scale: Ordinal

Result: a. Outdoor activity duration

1. < 30 minutes per day
2. 30 minutes-2 hours per day
3. >2 hours per day

b. Outdoor activity frequency

1. Never
2. 1-2 times per week
3. ≥ 3 times per week

7. Parental history of myopia

Definition: History of biologic father and or biologic mother with nearsightedness.

Scale: Nominal

Result: 1. Yes

2. No

8. Corneal curvature

Definition: The average of horizontal diameter and vertical diameter radius of cornea (in mm) for each eye. Corneal curvature will be measured by manual keratometer.

Scale: interval

Result: 1. Steeper cornea (≤ 7.8 mm)

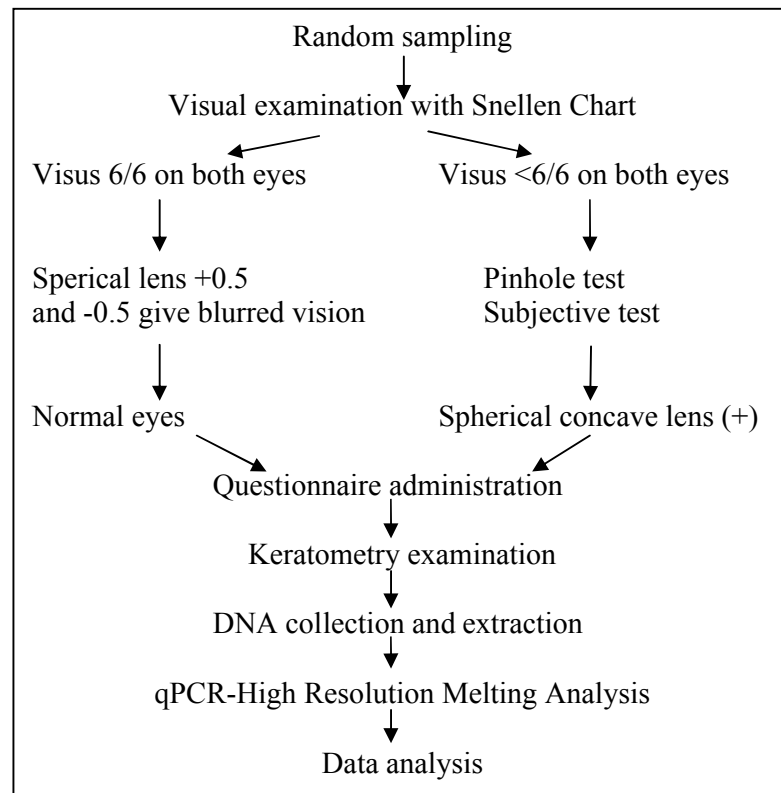
2. Flatter cornea (> 7.8 mm)

4.7 Data analysis

The data were analyzed by SPSS software version 17.0. The output data from SPSS were:

- a. Prevalence and distribution of *PDGFRA* gene polymorphism.
- b. Prevalence risk for risk factors of early-onset myopia, corneal curvature, and *PDGFRA* gene polymorphism.

4.8 Research flow



4.9 Research Ethics

The study protocol was submitted to the ethical committee of Medical Faculty of Muhammadiyah University, Palembang, South Sumatera, Indonesia.